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PRINCIPAL INVESTIGATOR: **Daniel W. Beury**

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Daniel W. Beury
Suzanne Ostrand-Rosenberg
email:b29@umbc.edu

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14. ABSTRACT

Previous studies have shown that MDSC block adaptive anti-tumor immunity by producing high levels of oxidizing agents such as reactive oxygen species (ROS), nitric oxide (NO), and peroxynitrite. Despite high levels of the toxic oxidizing agents, MDSC survive and suppress anti-tumor immunity. We hypothesize that MDSC survival is mediated by the antioxidant-regulating transcription factor Nrf2. To test this hypothesis, wild type and $Nrf2^{-/-}$ BALB/c and C57BL/6 mice were injected with 4T1 mammary carcinoma cells or MC38 colon carcinoma cells, respectively. Tumor-bearing mice were assayed weekly for percentage of MDSC in the blood and for MDSC levels of ROS and glutathione, MDSC production of H_2O_2 and suppressive activity, and MDSC apoptosis. Mice were also followed for survival. $Nrf2^{-/-}$ MDSC had more ROS and less glutathione than wild type MDSC, indicating that $Nrf2^{-/-}$ MDSC were more oxidatively stressed. $Nrf2^{-/-}$ MDSC were more apoptotic than wild type MDSC. $Nrf2^{-/-}$ and wild type mice had similar rates of primary tumor growth and MDSC accumulation, but tumor-bearing $Nrf2^{-/-}$ mice lived longer, indicating that Nrf2 contributes to tumor progression. $Nrf2^{-/-}$ MDSC produce less H_2O_2 and were less suppressive than wild type MDSC. These data are consistent with our hypothesis that Nrf2 regulates MDSC survival and suppressive activity, resulting in less suppressive MDSC in $Nrf2^{-/-}$ mice, thereby increasing anti-tumor immunity against metastatic disease.

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Introduction

Immune suppression is a major obstacle to breast cancer immunotherapy. A primary reason that immunotherapy is not effective is due Myeloid-Derived Suppressor Cells (MDSC). MDSC are a heterogeneous population of immature myeloid cells that accumulate in the blood, secondary lymphoid organs, and in primary and metastatic tumors in tumor-bearing individuals. MDSC are characterized by the surface markers Gr1 and CD11b in mice, and CD33 and CD11b in humans [1-3]. A variety of endogenous factors including vascular endothelial growth factor (VEGF) [4], prostaglandin E2 (PGE2) [5], IL-1β [6, 7], IL-6 [8], S100A8/A9 [9, 10], the complement component C5a [11], and endotoxin [12] induce the accumulation of MDSC. MDSC block adaptive anti-tumor immunity by inhibiting the activation of $CD4^+$ and $CD8^+$ T lymphocytes [2, 13, 14]. MDSC also produce IL-10, which polarize macrophages to a tumor prototing phenotype [15, 16]. A primary mechanism of MDSC-mediated suppression of T cells is by MDSC production of short-lived oxidants such as reactive oxygen species (ROS), nitric oxide, and peroxynitrite [17]. These reactive oxidizing agents are vital for T cell repression and for maintaining the inflammatory tumor microenvironment [18]. However, MDSC survive despite their high levels of these non-discriminatory toxic radicals. *I hypothesize that MDSC withstand these oxidizing agents due to the transcriptional regulator Nrf2.* Nrf2 is stabilized by the same factors that induce MDSC accumulation and suppression. Cells that are resistant to oxidative stress express the transcription factor NF-E2 related factor 2 (Nrf2). Nrf2 is considered a "good" transcription factor that when activated, induces the expression of protective and survival genes for antioxidant responses, phase II detoxification enzymes, and a plethora of other genes. These genes are grouped based on function and include genes for detoxification, antioxidant response, transcription, growth, defense and inflammatory response, signaling, and others [19, 20]. Nrf2 regulates genes controlled by the anti-oxidant response element (ARE) [21, 22] that are responsible for antioxidant responses, including glutathione synthesis genes such as GCL (Glutamate-Cysteine Ligase), and cystine transport genes x_CT and 4F2 [19, 23] Cystine transport and Nrf2 may contribute to MDSC survival. We have previously shown that MDSC sequester cysteine [24]. This sequestration may facilitate MDSC resistance to toxic radicals since importation of cystine (via the x_C^- cystine/glutamate antiporter) and its reduction to cysteine are rate-limiting for the synthesis of the antioxidant glutathione (GSH) in MDSC. Nrf2 is a major transcriptional regulator of x_C- and GSH synthesis genes [19, 20, 23]. Nrf2 is activated by the same oxidative radicals that MDSC use to facilitate immune suppression. Nrf2 protects cells against inflammation and is stabilized in response to inflammation, hypoxia, and other factors that are known inducers of MDSC. *Since Nrf2 regulates antioxidant response and apoptosis, I hypothesize that Nrf2 regulates MDSC survival by protecting MDSC from oxidative stress*. To test this hypothesis, I will be utilizing tumor-bearing Nrf2 deficient and wild type mice and comparing MDSC function and apoptotic rate in addition to monitoring these mice for survival and metastatic disease.

Aim 1: Determine if Nrf2 regulates MDSC survival by testing $Nrf2^{-/-}$ and wild type MDSC for apoptotic marker expression *in vivo* and the rate of apoptosis *in vitro*.

Aim 2: Determine if Nr2 regulates tumor-bearer survival and MDSC suppressive activity.

Aim 3: Determine if blocking cystine transport into MDSC while providing T cells with cysteine is a therapy for reducing MDSC-mediated immune suppression and delaying the growth of primary and metastatic mammary carcinomas.

Completion of these aims will determine if Nrf2 is a critical regulator of MDSC function and survival. New insight into Nrf2 modulating MDSC activity will provide future avenues for targeting MDSC as an adjuvant to cancer immunotherapy.

Body

Aim 1- In Progress

MDSC are functional immune suppressors despite their exposure to constant oxidative stress. Blood MDSC have high levels of ROS and tumor-infiltrating MDSC produce even more ROS (Previous data). Tumor-infiltrating MDSC are also exposed to even more oxidative stress due to the poorly vascularized and hypoxic tumor microenvironment and by ROS produced directly by tumor cells. Despite high levels of oxidative stress, MDSC are functionally suppressive and do not apoptose. Previously I had shown that MDSC from $Nrf2^{-/-}$ MDSC are more oxidativly stressed and apoptotic than wild type MDSC. *I hypothesize that MDSC resist apoptosis from oxidative stress by the activity of Nrf2.* If MDSC lack Nrf2, then they would be more susceptible to apoptosis from oxidative stress.

Aim 2- In Progress

MDSC are functional immune suppressors and tumor-bearer survival is negatively correlated with MDSC suppressive activity [6]. Previously I had shown that MDSC from 4T1 tumor-bearing wild type mice produced more H_2O_2 and were more suppressive than MDSC from 4T1 tumor-bearing $Nrf2^{-/-}$ mice. MDSC suppressive activity.

4T1 metastasis is the cause of death in 4T1 tumor-bearing animals [25] and 4T1-bearing $Nrf2^{-/-}$ animals may live longer due to enhanced resistance to metastasis. Resistance to metastasis requires a competent immune system [26]. Since $Nrf2^{-/-}$ MDSC are less suppressive, then there would be less immune suppression and could potentially allow for tumor-bearing $Nrf2^{-/-}$ mice to survive longer because they are more resistant to metastatic disease compared to tumor-bearing wild type animals. The following data support this hypothesis. For all of the following experiments, wild type and $Nrf2^{-/-}$ BALB/c and C57BL/6 mice were injected with 7000 4T1 tumor cells in the mammary fat pad or $5x10^5$ MC38 colon carcinoma cells in the flank, respectively.

Nrf2 does not impact primary tumor growth or MDSC accumulation, but decreases survival time of tumor-bearing mice. Since wild type MDSC are more suppressive and oxidativly stressed than $Nrf2^{-/-}$ MDSC, we hypothesized that Nrf2 would enhance tumor progression and MDSC accumulation of tumor bearing mice. However, we observed that Nrf2 did not impact primary tumor growth of 4T1 or MC38 (Figure 1A). Nrf2 did not impact the percentage of MDSC circulating in the peripheral blood. However, MDSC levels increased with increasing tumor burden (Figure 1B). Despite similar sizes of primary tumor and levels of MDSC, we observed that 4T1 and MC38 tumor-bearing $Nrf2^{-/-}$ animals live longer than their wild type counterparts indicating that Nrf2 decreases survival time of tumor-bearing mice (Figure 1C).

Aim 3-Completed

Aim 3 was completed during the 2010-2011 report period.

Key Research Accomplishments

Training Plan

Task 1: Meet yearly with my dissertation committee to review my experimental progress in the project. **(Completed to date)**

Task 2: Participate in weekly lab meetings, journal clubs, seminars, and talks with outside speakers. **(Completed to date)**

Task 3: Meet with my mentor weekly to discuss ongoing experiments. **(Completed to date)**

Task 4: Review manuscripts related to my proposal as suggested by my mentor. **(Completed to date)**

Task 5: Complete all necessary lab work to fulfill the objectives outlined in the research proposal. **(In progress)**

Task 6: Complete coursework required by the Biological Sciences Ph.D. program. **(Completed)**

Task 7: Pass oral examination on the background of my research, present and successfully defend my research during the comprehensive preliminary/qualifying exam to pass onto Ph.D. candidacy. **(Completed)**

Task 8: Present my research at minimum of one national conference per year. **(Completed to date)**

Task 9: Write up experimental results in a timely manner for publication in peer-reviewed journals.

Task 10: Collaborate with other students and investigators to fulfill my objectives. **(In progress)**

Task 11: Serve as a teaching assistant for two semesters. **(Completed)**

Task 12: Present a departmental seminar describing my completed thesis project, and defend my Ph.D. dissertation before my dissertation committee. **(In Progress)**

Task 13: Locate a suitable post-doctoral position for continuation of my training. **(In Progress)**

Milestones and Deliverables:

1. Completion of my preliminary/qualifying exam. **(Completed)**

2. Completion of required coursework to fulfill the Biological Sciences Ph.D. program. **(Completed)**

3. Complete two semesters as a teaching assistant. **(Completed)**

4. Present my first oral presentation at a national conference.

5. Have my thesis research published in a well-respected, peer reviewed journal. **(In Progress)**

6. Successfully defend my Ph.D. dissertation. **(In Progress)**

7. Obtain an appropriate and well-regarded post-doctoral position. **(In Progress)**

Task 1: Determine if Nrf2 regulates MDSC survival. (In Progress)

Task 1A: Determine the rate of cell death of $Nrf2^{-/-}$ MDSC compared to wild type MDSC. **(Completed)**

Task 1B: Determine if Nrf2 regulates GSH levels and MDSC apoptosis in response to oxidative stress.

Task 1C: To determine if GSH regulates apoptosis in MDSC.

Task 1D: Determine if tumor MDSC more susceptible to apoptosis than blood MDSC.

Task 1E: Determine if Nrf2 protects MDSC from the oxidative tumor microenvironment.

Task 1F: Determine if Nrf2 protects MDSC from hypoxia.

Outcomes/Products/Deliverables: Nrf2 enhances MDSC resistance to apoptosis.

Task 2: Determine if Nrf2 regulates tumor-bearer survival and MDSC suppressive activity.

Task 2A: Determine if Nrf2 regulates ROS, NO, and peroxynitrite production in MDSC. **(In Progress)**

Task 2B: Determine if Nrf2 regulates the suppressive activity of MDSC. **(In Progress)**

Task 2C: Determine if Nrf2 regulates MDSC accumulation and mammary tumor growth. **(Completed)**

Outcomes/Products/Deliverables: Nrf2 decreases MDSC oxidative stress and increases MDSC production of ROS, MDSC suppressive activity, and survival of tumor-bearing mice. Nrf2 does not impact tumor growth or MDSC accumulation in tumor-bearing mice. MDSC do not produce reactive nitrogen species.

Task 3: Determine if inhibition of MDSC sequestration of cysteine (via xCT) reduces MDSC accumulation, restores immune competence, delays metastatic disease, and increases survival time. **(Completed)**

Task 3A: Determine if SASP and NAC reduce MDSC production of ROS, NO, peroxynitrite, GSH levels, cystine transport, and reduce MDSC resistance to Fas-mediated apoptosis and suppressive activity. **(Completed)**

Task 3B: Determine if SASP and NAC affect MDSC accumulation and mammary tumor growth. **(Completed)**

Outcomes/Products/Deliverables: SASP reduces MDSC viability, GSH content, and cystine transport *in vitro*. There is no difference between inflammation-induced and conventional MDSC transport of cystine. SASP has no effect on tumor growth, metastatic disease, MDSC accumulation, or MDSC suppressive activity.

Reportable Outcomes

Milestones and Deliverables:

- Completed my preliminary/qualifying exam.
- Confirmed that Nrf2 regulates MDSC accumulation.

Presentations:

- Daniel W. Beury, Cassandra Nelson, Suzanne Ostrand-Rosenberg "Transcription Factor Nrf2 (NF-E2 Related Factor 2) Enhances Myeloid-Derived Suppressor Cell (MDSC) Accumulation and Tumor Progression" American Association of Immunologists 99th Annual Meeting. Boston, MA. May 4-8, 2012 (poster presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg "Communication among tumor-infiltrating immune cells enhances tumor-progression" UMBC Biological Sciences Departmental Seminar, Nov 28th, 2012 (oral presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg, "Anti-inflammatory effects of Myeloid-Derived Suppressor Cells and Macrophage crosstalk contribute to tumor progression" American Association for Cancer Research Annual Meeting. Washington, DC. April 6-10, 2013
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg, "Anti-inflammatory effects of Myeloid-Derived Suppressor Cells and Macrophage crosstalk contribute to tumor progression" American Association of Immunologists 100th Annual Meeting. Honolulu, Hawaii. May 3-7, 2013

Conclusions to Date

- It has been demonstrated that Nrf2 regulates oxidative stress in MDSC and MDSC apoptosis. Research to ascertain which proteins downstream of Nrf2 mediate MDSC oxidative stress and apoptosis would provide novel targets for future therapies aimed at reducing MDSC levels in tumor-bearing patients for enhancement of immunotherapeutic strategies of targeting cancer.
- It has been shown that Nrf2 does not increase MDSC accumulation in tumor-bearing animals or affect primary tumor growth, but does reduce survival in tumor-bearing animals. It has also been shown that Nrf2 enhances MDCS suppressive mechanisms and MDSC suppressive activity. Research to ascertain the mechanisms of Nrf2's pro-tumor activity and enhancement of MDSC suppressive activity would provide novel pathways to increase anti-tumor immunity.
- It has been demonstrated that inflammation enhances x_C- expression on MDSC, but higher x_C- expression does not enhance the ability of MDSC to transport cystine. *In vitro,* SASP inhibits cystine transport, reduces intracellular GSH, and increases cell death in MDSC. However, therapeutic administration of oral sulfasalazine to tumor-bearing animals has no effect on primary tumor growth, MDSC accumulation, metastatic disease, or MDSC suppressive activity. Therefore, SASP is a poor candidate for treatment of tumor-bearing individuals.

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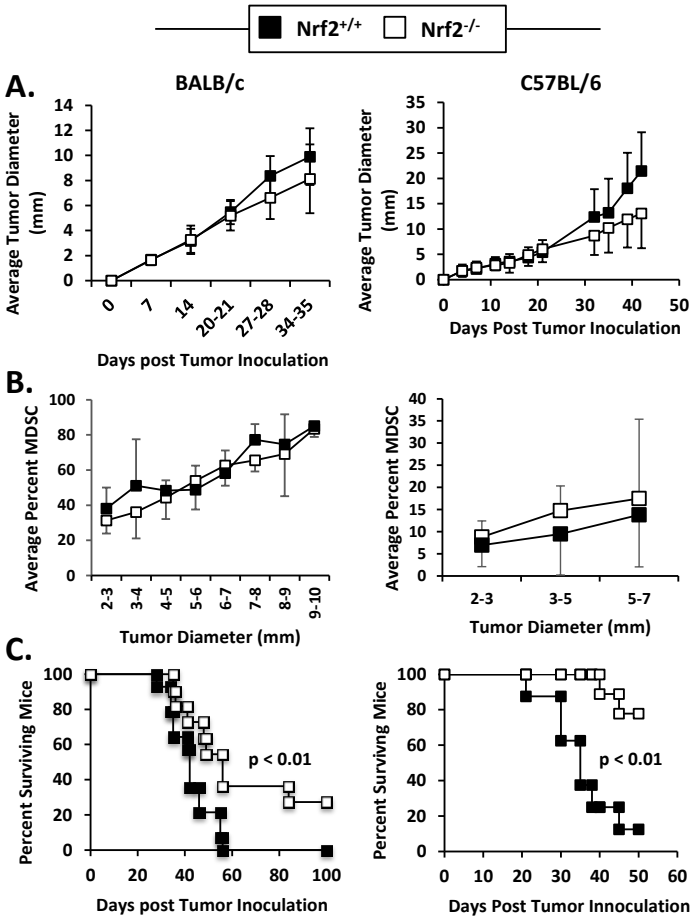
Supporting Data

Figure 1: Figure 5: Nrf2 does not impact primary tumor growth or MDSC accumulation, but decreases survival time of tumor-bearing mice.



Groups of $Nrf2^{-/-}$ and wild type mice from BALB/c (Left panels) and C57BL/6 (Right panels) backgrounds were injected with 4T1 and MC38, respectively. BALB/c $Nrf2^{+/+}$ n=14; BALB/c $Nrf2^{-/-}$ n=11; C57BL/6 $Nrf2^{+/+}$ n=8; C57BL/6 $Nrf2^{-/-}$ n=9. Data from BALB/c mice was pooled from two independent experiments. **(A)** Nrf2 does not impact primary tumor growth. Tumor bearing mice were monitored weekly for primary tumor growth. Data was analyzed by *t*-test. **(B)** Nrf2 does not impact MDSC accumulation. Tumor-bearing mice were bled weekly to determine the percentage of MDSC by flow cytometry and MDSC percentage was plotted as a function of primary tumor diameter. Data was analyzed by Mann-Whitney test. **(C)** Nrf2 decreases survival time of tumor bearing mice. Tumor bearing mice were monitored for survival time. Data was analyzed for significance by log-rank test.